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Characterization and Nucleotide Sequence
Determination of a Repeat Element Isolated from a
2,4,5-T Degrading Strain of 'Pseudomonas cepacia'

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Characterization and nucleotide sequence determination of a repeat element isolated from a 2,4,5-T degrading strain of *Pseudomonas cepacia*

(Gene evolution; Tn5 mutagenesis; insertion sequence; 2,4,5-trichlorophenoxyacetic acid; chlorohydroxyhydroquinone metabolizing gene; genetic homology; DNA probe)

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SUMMARY

Pseudomonas cepacia strain AC1100, capable of growth on 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), was mutated to the 2,4,5-T⁻ strain PT88 by a ColE1::Tn5 chromosomal insertion. Using cloned DNA from the region flanking the insertion, a 1477-bp sequence (designated RS1100) was identified which was repeated several times on the wild-type chromosome and was also present on AC1100 plasmid DNA. Various chromosomal fragments containing this sequence were cloned and their nucleotide sequence was determined. Examination of RS1100 revealed the presence of 38-39-bp terminal inverted repeats immediately flanked by 8-bp direct repeats. The translated sequence of the single large open reading frame of RS1100 showed structural similarity to the phage Mu transposase and other DNA-binding proteins. Thus the AC1100 repeated sequence has several structural features in common with insertion sequence elements. Three copies of RS1100 were mapped near 2,4,5-*t* genes encoding degradation of 5-chloro-1,2,4-trihydroxybenzene, an intermediate in 2,4,5-T degradation. Neither RS1100 nor the 2,4,5-*t* genes hybridized to DNA isolated from *Pseudomonas* strains, including *P. cepacia*, suggesting that both gene fragments may be of foreign origin recruited in strain AC1100. The origin of these two DNA segments as well as the role played by RS1100 in the recruitment of 2,4,5-*t* genes in AC1100 are presently under investigation.

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Abbreviations: Ap, ampicillin; bp, base pair(s); BSM, basal salts medium (see Kilbane et al., 1982); CHQ, chlorohydroxyhydroquinone; *chq*, gene specifying CHQ metabolism; Δ , deletion; DR, direct repeat; IS, insertion-sequence element; ITR, inverted terminal repeat; kb, 1000 bp; Km, kanamycin; LB, Luria-Bertani medium; NAH, naphthalene plasmid; Nal, nalidixic acid; nt, nucleotide(s); ORF, open reading frame; RS1100, repeated sequence element from AC1100; ^R, resistance; Sm, streptomycin; Tc, tetracycline; Tn, transposon; TOL, xylene/toluene plasmid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; 2,4,5-*t*, gene specifying the degradation of 2,4,5-T; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; [], designates plasmid-carrier state.

INTRODUCTION

P. cepacia strain AC1100, capable of utilizing 2,4,5-T as its sole source of carbon and energy, was isolated from a mixed bacterial population exposed to increasingly stringent 2,4,5-T selection pressure in a chemostat (Kilbane et al., 1982). The evolution of novel catabolic pathways has been attributed to several molecular mechanisms. These include recruitment and expression of foreign genes, especially those carried on catabolic plasmids, and various genetic rearrangements (deletions, chromosome/plasmid fusions, gene duplication). Repeated sequences have been implicated as sites for homologous recombination between bacterial plasmids and chromosomes (Barsomian and Lessie, 1986; Szabo and Mills, 1984), deletion formation (Kaluza et al., 1985), and genetic rearrangements (gene duplication, translocation) leading to evolution of new enzymes for degradation of novel synthetic compounds (Negoro et al., 1983). Repeated sequences have been discovered in many prokaryotic systems including Gram-negative and Gram-positive eubacteria, as well as the archaebacteria (Sapienza et al., 1982; Fishman et al., 1985). Other repeated sequences are actually transposable IS elements which have been associated with gene mutation, deletions, replicon fusions and activation of gene expression (Comai and Kosuge, 1983; Gay et al., 1985; Scordilis et al., 1987).

To conduct a genetic analysis of the 2,4,5-T pathway, mutants which were incapable of growth on 2,4,5-T were generated from AC1100 ColE1::Tn5 transconjugants (Tomasek et al., 1986). One such mutant, PT88, was used for isolation of 2,4,5-T-degradative genes involved in the metabolism of CHQ, an intermediate in the degradation of 2,4,5-T (Sangodkar et al., 1988). On subsequent cloning of the regions flanking the Tn5-insertion site in PT88, we discovered an element which was repeated several times on the parental AC1100 genome. Three copies of this element have also been shown to flank 2,4,5-T genes cloned on a 25-kb fragment. In this paper we report identification and cloning of these repeating elements (designated as RS1100) from *P. cepacia* AC1100 and describe a complete nucleotide sequence. Additionally, we also demonstrate that this element, as well as the flanking 2,4,5-T genes, are foreign to the *P. cepacia* background, and may have been recruited from a common ancestor.

MATERIALS AND METHODS

(a) Bacterial strains, plasmids and growth media

Pseudomonas strains, *Escherichia coli* strains, and plasmids used are listed in Table I. *P. cepacia* 249 was obtained from T. Lessie. *E. coli* strains were grown at 37°C on Luria-Bertani (Maniatis et al., 1982) or on glucose-free medium (Lennox broth base, Gibco Laboratories), supplemented with 1.5% agar for growth on plates. Antibiotics (Sigma Chemical Co.) used were 50 µg Ap/ml; 50 µg Km/ml; and 15 µg Tc/ml.

All *Pseudomonas* strains were grown at 30°C. *P. cepacia* strain AC1100 was grown on 1 mg/ml of 2,4,5-T (Aldrich), in BSM (Kilbane et al., 1982). BSM supplemented with 1.5% Noble agar (Difco Laboratories) was used for plates. AC1100 cells used for plasmid and chromosomal DNA isolations were grown in 1 liter of 2,4,5-T mineral salts medium in 2-liter erlenmeyer flasks shaken at 250 rev./min. Other *Pseudomonas* strains were grown on LB, *Pseudomonas* isolation agar (Difco) or BSM containing 0.2% glucose.

(b) Plasmid and chromosomal DNA isolation

All chemicals were from Sigma Chemical Co., unless otherwise noted. Plasmid DNA was isolated by a modification (Chatterjee et al., 1981) of the method described by Casse et al. (1979). Chromosomal DNAs for *P. aeruginosa*, *P. mendocina*, *Azotobacter vinelandii*, *Arthrobacter viscosus*, *Klebsiella pneumoniae*, and *E. coli* were provided by A. Darzins (Darzins et al., 1986).

(c) Restriction digestions, isolation of DNA fragments, ligations and transformation

All restriction enzymes were purchased from New England Biolabs or Bethesda Research Labs and used according to the manufacturers' directions. DNA fragments were separated by electrophoresis on 0.7% agarose gels. Individual DNA fragments were extracted from gels by electrophoresing bands onto DEAE membranes (NA45; Schleicher & Schuell, Inc.) and eluting them according to the manufacturer's instructions. T4 ligase was purchased from New England Biolabs. Ligations were performed at 12°C, overnight. Synthetic *SalI* linker

TABLE I

Bacterial strains and plasmids

Strains or plasmids	Characteristics	Reference
Strains		
<i>E. coli</i>		
AC80	<i>thr leu met hsdR, hsdM</i>	Chakrabarty et al. (1978)
HB101	<i>recA hsdS20 (hsdR⁻ hsdM⁻) pro rpsL20 (Sm^R)</i>	Bolivar et al. (1977)
DH1	<i>recA hsdR17 (hsdR⁻ hsdM⁺) gyrA9(supE</i>	Maniatis et al. (1982)
TB1	<i>hsdR⁻ hsdM⁺ ara Δ(lac-pro) rpsL20 (Sm^R) thi φ80dlacZ ΔM15</i>	Bagdasarian and Timmis (1982)
<i>P. cepacia</i>		
AC1100 (DC102)	2,4,5-T ⁺ , Nal ^R	Chatterjee et al. (1982)
PT88 and other Tn5 induced mutants of AC1100 (Fig. 7)	2,4,5-T ⁻ , Nal ^R , Km ^R	Sangodkar et al. (1988); Tomasek et al. (1986)
Spontaneous mutants of AC1100: 9A11, 9A12, 6B32, 12B2, 7C1, 8C1 (Fig. 7)	2,4,5-T ⁻	This study
Plasmids		
pRK2013	ColE1, <i>mob⁺ tra⁺</i> (RK2), Km ^R	Figurski and Helinski (1979)
pRZ102	ColE1 : : Tn5, Km ^R	Jorgensen et al. (1979)
ps1EMBL	4.2 kb, R6K, Km ^R <i>lacZ'</i>	Poustka et al. (1984)
pBR322	4.4 kb, ColE1 Tc ^R Ap ^R	Bolivar et al. (1977)
pPT121	38 kb, ColE1 Ap ^R Km ^R , pHC79 cosmid clone of PT88 total DNA including Km ^R gene from ColE1 : : Tn5 chromosomal insertion	This study
pPT122	42-kb, ColE1 Ap ^R Km ^R , same description as pPT121	This study
pPT128	10.4-kb, ColE1 Ap ^R Km ^R 6-kb <i>SalI</i> fragment from pPT121 cloned into <i>SalI</i> site of pBR322	This study
pPT129	10.2-kb, R6K, Km ^R , 6-kb <i>SalI</i> fragment from pPT121 cloned into <i>SalI</i> site of ps1EMBL	This study
pPT165	5.5-kb, R6K, Km ^R , 1.3-kb <i>SalI-PvuII</i> fragment of pPT130 with <i>PvuII</i> end ligated to a <i>SalI</i> linker cloned into <i>SalI</i> site of ps1EMBL	This study
pPT181	6.2-kb, R6K, Km ^R , 2.1-kb <i>EcoRI</i> fragment from AC1100 chromosomal DNA cloned into <i>EcoRI</i> site of ps1EMBL	This study
pPT182	10.9-kb, R6K Km ^R , 3.2-kb <i>EcoRI</i> fragment including 2.0 kb <i>EcoRI-HindIII</i> piece) and 3.5-kb <i>EcoRI</i> fragment cloned into <i>EcoRI</i> site of ps1EMBL	This study
pCP13	21-kb Tc ^R Km ^R <i>cos⁺</i>	Darzins et al. (1986)
pHC79	6.4-kb Tc ^R Ap ^R <i>cos⁺</i> ColE1, pBR322	Hohn and Collins (1980)
pUS1 ^a	46-kb Tc ^R , complements PT88 mutation to 2,4,5-T ⁺	Sangodkar et al. (1988)
pUS105 ^a	29-kb Tc ^R , complements PT88 mutation to 2,4,5-T ⁺	Sangodkar et al. (1988)
pUS1025 ^a	24-kb Tc ^R , 3-kb <i>BamHI-HindIII</i> carrying RS1100	This study
pUS1028 ^a	27.7-kb Tc ^R , complements PT88 mutation to 2,4,5-T ⁺	Sangodkar et al. (1988)
pUS1029 ^a	25.3-kb Tc ^R , complements PT88 mutation to 2,4,5-T ⁺	Sangodkar et al. (1988)

^a pUS1 to pUS1029 carry 25-kb to 3-kb insert in the 21-kb vector pCP13.

was purchased from Pharmacia. Competent *E. coli* TB1 or DH1 cells were transformed with ligated DNA using a CaCl_2 heat-shock treatment (Maniatis et al., 1982). Cells were then plated on selective media containing appropriate antibiotics. Cells carrying inserts in the LacZ α peptide gene in the multiple cloning site of ps1EMBL or pUC plasmids were selected based on the white colony color on XGal selection plates.

(d) Tn5 mutagenesis of strain AC1100

Plasmid pRZ102 (Jorgensen et al., 1979) containing ColE1::Tn5 was transferred from *E. coli* HB101 hosts to *P. cepacia* AC1100 by triparental matings with *E. coli* HB101[pRK2013]. *E. coli* strains were grown overnight in the LB medium with Km. AC1100 cells were grown as described above. Equal volumes of *E. coli* strains (0.5 ml) and 5 ml of *P. cepacia* AC1100 were filtered on a sterile membrane filter (HA, 0.45 μm pore size, Millipore) followed by passing of 0.5 ml of sterile BSM. The filters were placed on LB agar and incubated at 30°C for 15 h. The cells were washed from the filters by vortexing them with 0.5 ml BSM. Aliquots (0.1 ml) of the cell suspension were plated on individual selective plates (*Pseudomonas* isolation agar with 50 μg Km/ml). Plated cells were allowed to grow at 30°C for three days. Km^R colonies were tested for growth on 2,4,5-T plates supplemented with 50 μg Km/ml.

(e) Cosmid cloning

PT88 total chromosomal DNA was partially digested with *Sau*3A, under carefully controlled conditions so as to yield fragments 30 to 50 kb in size (Maniatis et al., 1982). These random chromosomal fragments were ligated to the *Bam*HI-digested cosmid vector pHC79. The DNA mixture was then packaged in vitro using a lambda packaging kit (Amersham) according to the manufacturer's instructions. The packaging mixture was used to transfect *E. coli* strain DH1 cells which were then plated on selective medium containing 50 μg Km/ml.

(f) Southern blot and colony hybridization analyses

In all procedures, nitrocellulose membranes (BA85; Schleicher & Schuell) were used for DNA

immobilization. DNA hybridization probes were prepared using components and instructions of a nick-translation kit (Bethesda Research Labs) with [³²P]dCTP (3000 $\mu\text{Ci}/\text{mmol}$; Amersham). Following agarose gel electrophoresis, Southern blots were performed as described in Maniatis et al. (1982). Partial depurination of DNA in the gel was accomplished by exposure to 0.25 M HCl, followed by two distilled water rinses before the denaturation step.

Colony hybridization was performed as described previously (Deretic et al., 1986). Background hybridization was reduced by gently brushing the lysed colonies with glove-protected fingers to remove cell debris following fixation of DNA to the membrane.

(g) Sequencing and computer-assisted analysis

Fragments from pPT165, pPT181, and pPT182 were subcloned in M13mp19 and sequenced using methods described previously (Frantz et al., 1987). The Intelligenetics SEQ program (Intelligenetics, Palo Alto, CA) was used to translate the nucleotide sequence. The Intelligenetics PEP program was used to predict potential protein secondary structures (according to the Chou and Fasman, 1978, algorithm).

RESULTS AND DISCUSSION

(a) Insertion mutagenesis of strain AC1100

Insertion mutagenesis of *P. cepacia* strain AC1100 resulted in the generation of approx. 90 mutants deficient in 2,4,5-T metabolism out of 1700 putative Km^R Tn5 insertion mutants screened. Various auxotrophic mutants requiring leucine, serine, methionine, or arginine were also isolated. Southern blots of *Eco*RI-digested total and plasmid DNAs from four 2,4,5-T⁻ mutants and a leucine auxotroph were probed with the 3.4-kb *Hind*III fragment of pRZ102 carrying Tn5 (Mazodier et al., 1985) to identify the sites of insertion. The probe hybridized only to the chromosomal DNAs in all cases (not shown).

(b) Cosmid cloning of PT88 chromosomal sequences flanking the Tn5 insertion site

One *P. cepacia* AC1100 2,4,5-T⁻ insertion mutant, designated PT88, accumulated a bright red

compound when grown on glucose in the presence of 2,4,5-T. This compound has been shown to be an oxidation product of CHQ (or 5-chloro-1,2,4-trihydroxybenzene), an intermediate in 2,4,5-T degradation (Sangodkar et al., 1988). Cosmid cloning of random *Sau*3A fragments from PT88 chromosomal DNA resulted in two Km^R *E. coli* colonies carrying plasmids, pT121 and pT122. When Tn5 (3.4-kb *Hind*III fragment of pRZ102) was used as a hybridization probe, both the plasmids were found to share a common 6-kb *Sal*I restriction fragment with the original PT88 chromosome. This fragment was subcloned from pPT121 into the *Sal*I site of pBR322 generating the recombinant plasmid pPT128. The 6-kb *Sal*I fragment carried 2.7-kb of Tn5 and expressed Km^R gene. Plasmid pPT129 was constructed by subcloning the same fragment into the *Sal*I site of pslEMBL, a cloning vector which does not have homology to pBR322-based plasmids (Poustka et al., 1984).

(c) Identification of nucleotide sequences from AC1100

The 6-kb *Sal*I fragment from subclone pPT129 was used as a hybridization probe against native AC1100 chromosomal DNA restriction digests in an effort to identify the region homologous to PT88 sequences flanking the Tn5 insertion site (on or near a 2,4,5-*t* gene since insertion of Tn5 produced a 2,4,5-T⁻ phenotype). Surprisingly, this probe hybridized to several fragments of AC1100 as well as PT88 chromosomal digests (Fig. 1A), indicating that there are certain sequences on the 6-kb fragment that are repeating on AC1100 genome. The 6-kb probe also hybridized to a number of fragments from plasmid DNA of AC1100. We further localized a 1.3-kb *Sal*I-*Pvu*II fragment distal to the Tn5 sequences of the 6-kb fragment and subcloned it as 1.3-kb *Sal*I fragment in pslEMBL, after addition of synthetic *Sal*I linker to the blunt *Pvu*II end (pPT165). The 1.3-kb fragment isolated from pPT165 was used as probe for Southern blots and colony hybridizations. The results (Fig. 1B) confirmed the repetition of several copies of this probe on the AC1100 genome. This 1.3-kb fragment did not hybridize to Tn5, pslEMBL or any ColE1-based plasmids. Chromosomal DNAs from *P. cepacia* 249, *P. cepacia* 383, *P. cepacia* 717, *P. aeruginosa*,

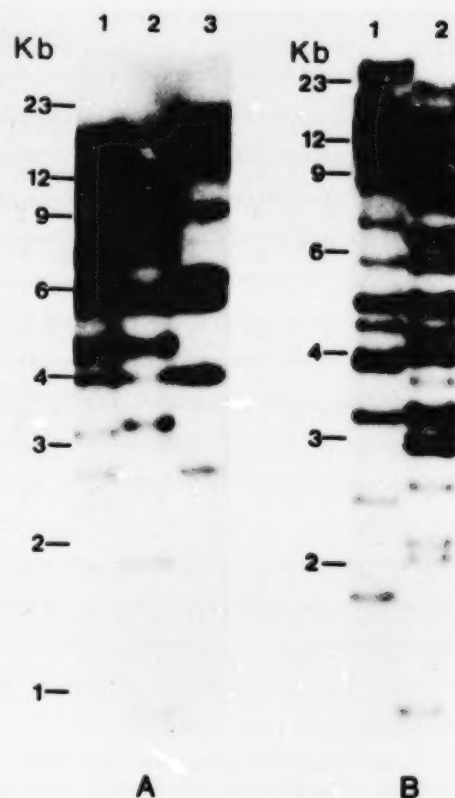


Fig. 1. DNA/DNA hybridization patterns of restriction endonuclease fragments from strain AC1100 DNA probed with isolated fragments containing the repeated sequence (RS1100). (Panel A) Lanes: 1–3, AC1100 and PT88 chromosomal DNAs and AC1100 plasmid DNA, respectively, digested with *Pvu*II. The hybridization probe was the 6-kb *Sal*I fragment cloned from PT88 in pPT129. (Panel B) Lanes: 1 and 2, AC1100 chromosomal DNAs digested with *Bam*HI and *Eco*RI, respectively. The probe was the 1.3-kb *Sal*I-*Pvu*II fragment cloned from PT88 in pPT165.

P. putida, *P. mendocina*, *Alcaligenes eutrophus* JMP134, *Azotobacter vinelandii*, *Arthrobacter viscosus*, *Klebsiella pneumoniae* and *E. coli*, as well as the colony blots from various soil isolates which were selected on *Pseudomonas* Isolation agar, did not show any homology to this probe, indicating that it is unique to *P. cepacia* AC1100 (see later).

(d) Cloning of AC1100 fragments containing the repeating elements

To further study the structure of the repeating element on the 1.3-kb fragment and its homologous

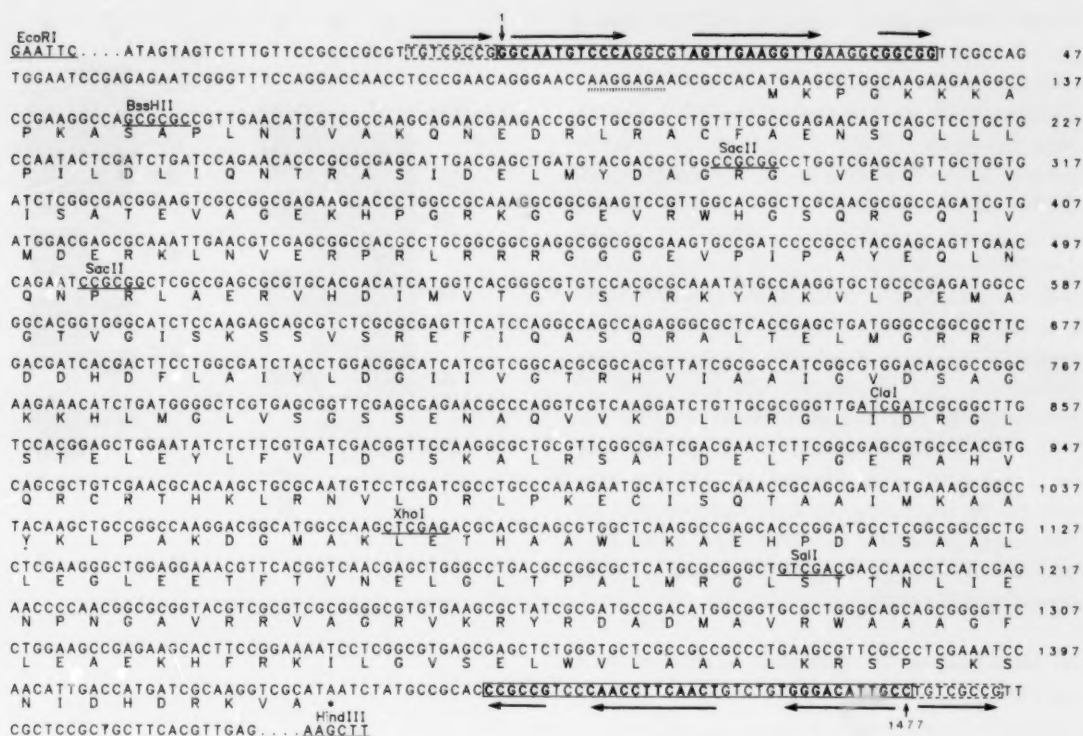


Fig. 2. Complete nucleotide sequence of RS1100. Clusters of ITRs are indicated by solid boxes. Flanking 8-bp DRs are designated by dashed boxes. Long arrows show the orientation of the repeated sequences. The amino acid (single-letter abbreviation) sequence translated from the large ORF is aligned below the nucleotide sequence. A putative Shine-Dalgarno ribosome-binding site is underlined by short vertical lines starting -15 bp from the start codon. Key restriction sites are indicated above the nucleotide sequence. Nucleotide numbering begins with the 'left' ITR at the downward arrow and ends at the 'right' ITR at nt position 1477 (upward arrow). The source of this sequence was the 2.4-kb *EcoRI*-*HindIII* AC1100 chromosomal fragment subcloned from the 3.2-kb *EcoRI* fragment of pPT182.

copies on AC1100 genome, we sought to randomly isolate such elements from AC1100 DNA. *EcoRI* fragments (approx. 2–4 kb size range) of AC1100 chromosomal DNA were purified from agarose gels and ligated to pslEMBL. Of the 50 recombinant plasmids screened, two positive clones were selected by colony hybridization with the 1.3-kb probe. The recombinant plasmids, pPT181 and pPT182, containing 2.1- and 3.2-kb *EcoRI* inserts, respectively, were selected. Plasmid pPT182 also contained a second *EcoRI* fragment (3.5 kb) which did not hybridize to the probe.

(e) Sequencing of the repeat elements

A 2.4-kb *EcoRI*-*HindIII* fragment of pPT182 which hybridized to the 1.3-kb probe was cloned into

M13mp19 for DNA sequencing. After close analysis of the nucleotide sequence of this fragment, a 1477-bp region was located which had distinct structural features of an insertion element (Fig. 2). These features include: 8-bp DRs on either end of the sequence, followed by imperfect inverted repeats (38 or 39 bp in length). The 'left' ITR contains three clusters (12 + 12 + 6 nt) which are exactly complementary to those from the 'right' terminal repeat interspersed with 3–5 nt which are not homologous. ITR sequences with such gaps are characteristic of many IS elements (Kleckner, 1981). The nucleotide sequence, including the two ITRs and the intervening nucleotide, is designated as RS1100.

The 2.1-kb *EcoRI* fragment of pPT181 and the 1.3-kb *SalI* fragment of pPT165 were similarly sequenced after subcloning into respective sites of

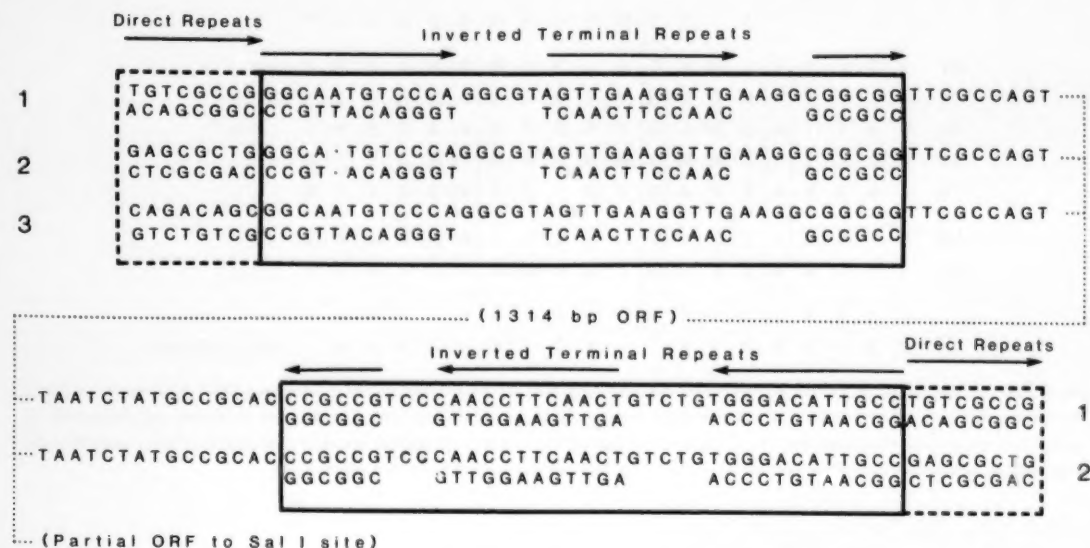


Fig. 3. ITRs of RS1100 and flanking DRs sequenced from various chromosomal fragments. For clarity, both strands of the ITRs and DRs are shown, whereas the unmatched sequences between the 'left' (upper) and 'right' (lower) ITRs are indicated by the single strand. Sequence 1 is the same as that shown in Fig. 2. The source of sequence 2 was the 2.1-kb *EcoRI* AC1100 chromosomal fragment cloned in pPT181. Although indicated by dotted lines, the complete nucleotide sequence of 2 was determined and was identical to sequence 1 with the following exception: the 'left' ITR of sequence 2 was missing an A-T pair which is present in the other two sets of ITRs sequenced. The source of sequence 3 was the 1.3-kb *PvuII-SalI* fragment from PT88 chromosome cloned in pPT165. The 'right' ITR and DR from sequence 3 were not included in this clone and were not sequenced. The designation of the 'left' DR and ITR of sequence 3 must therefore be regarded as tentative.

M13mp19. Comparison of the nucleotide sequences of the RS1100s from the three fragments (Fig. 3), indicates that the ITRs and the intervening sequences match exactly, except one missing A-T pair in the leftmost inverted repeat of RS1100 of 2.1-kb fragment of pPT181. This may represent a variant form of RS1100, since the two other terminal repeat sets sequenced have two A-T pairs at that location. An interesting observation is the presence of three different direct repeats (8 bp) on both sides of each of the RS1100s of three fragments (Fig. 3). Flanking direct repeated sequences are generally associated with typical IS elements, wherein one of the direct repeats arises from the original site of insertion (target DNA) and the other is a result of a duplication event during transposition (Grindley and Reed, 1985). In strain AC1100, therefore, the putative IS element (RS1100) may not require specific target sequences for transposition.

Another feature of many IS elements is the presence of a large ORF which codes for a transposase (Kleckner, 1981). The polypeptide predicted from the large ORF of RS1100 shows a fair degree

of homology to the secondary structures of phage Mu transposase and other DNA-binding proteins (Fig. 4). The DNA-binding sites of such proteins are characterized by a helix-turn-helix motif (Harshey et al., 1985; Pabo and Sauer, 1984). The alanine, glycine, and valine or isoleucine residues are highly conserved residues within the helix-turn-helix. The Chou and Fasman (1978) computer-predicted secondary structures for both Mu transposase and RS1100 polypeptide at the putative DNA-binding site are shown in the lower part of Fig. 4, along with the conserved alanine, glycine and valine residues.

(f) Localization of RS1100 on cloned fragment carrying genes involved in 2,4,5-T degradation

To determine whether copies of RS1100 are directly located near the 2,4,5-*t* genes, we screened cloned genes complementing the 2,4,5-*T*⁻ mutation in PT88. Plasmid pUS1 carries a 25-kb insert of AC1100 chromosomal DNA (Fig. 5). A 4.3-kb region of this insert harbors the *chq* genes responsible for the metabolism of CHQ or 5-chloro-1,2,4-trihy-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
282	I	T	E	I	A	L	D	Y	G	F	L	H	L	G	R	F	A	E	N	Y	XylS
16	Q	T	K	T	A	K	D	L	G	V	Y	Q	S	A	I	N	K	A	I	H	λ Cro
28	R	A	E	I	A	Q	R	L	G	F	R	S	P	N	A	A	E	E	H	L	Lex R
33	Q	E	S	V	A	D	K	M	G	M	G	Q	S	G	V	G	A	L	F	N	λ R
6	L	Y	D	V	A	E	Y	A	G	V	S	Y	Q	T	V	S	R	V	V	N	Lac R
389	P	V	E	R	A	F	G	V	G	G	L	E	E	Y	V	D	K	H	P	A	Mu A
154	L	P	E	M	A	G	T	V	G	I	S	K	S	S	V	S	R	E	F	I	RS1100
	A	A	A	A	A	A	T	T	T	T	A	A	A	A	A	A	A	A	A	A	Mu A structure
	A	A	A	A	A	A	A	A	-	-	T	T	T	T	-	A	A	A	A	A	RS1100 structure

Fig. 4. Primary and predicted secondary protein structure of large RS1100 peptide compared to proposed DNA binding site of phage Mu transposase (Mu A) and regions of other proteins having known DNA-binding sites. The primary structures are designated by single-letter amino acid abbreviations. The Chou and Fasman (1978) computer-predicted secondary structures of Mu A and RS1100 large peptide are shown with A's representing α -helical regions and T's representing turn regions.

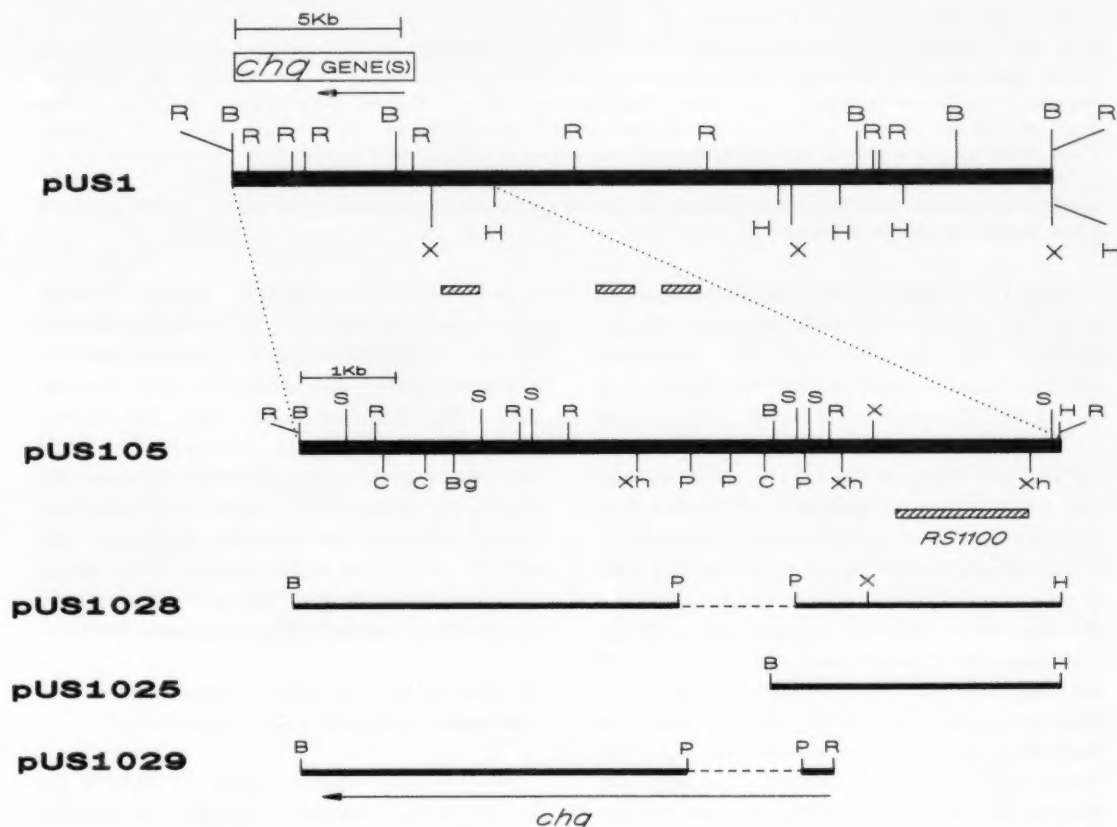


Fig. 5. Restriction map of 25-kb insert of pUS1 indicating the location of RS1100. The open box shows the position of 2,4,5-*t* genes (*chq*) involved in the metabolism of CHQ. Hatched boxes indicate the location of RS1100. Extended map of pUS105 and its deleted derivatives shows the position of one RS1100 copy which was cloned on 3-kb *Bam*II-*Hind*III fragment in pUS1025. The restriction sites are abbreviated as: B, *Bam*HI; Bg, *Bgl*II; C, *Cl*aI; H, *Hind*III; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I; Xh, *Xho*I. Arrow shows direction of transcription. Dashed lines, deletions.

droxybenzene, a metabolite of 2,4,5-T degradation (Sangodkar et al., 1988). We used ^{32}P -labeled 1.3-kb *Sal*I fragment of pPT165 carrying RS1100 for hybridization. The Southern-blot analysis of *Sal*I and *Eco*RI restriction digests of the 25-kb insert of pUS1 showed that RS1100 hybridized with 2.4, 2.38 and 1.4-kb *Sal*I fragments and 5.1- and 4.8-kb *Eco*RI fragments (Fig. 6). Further analysis of pUS1 digests indicated that the 4.8-kb *Eco*RI fragment contains two copies of RS1100 (results not shown). Fig. 6 also shows the profiles of hybridization experiments with restriction digests of pUS105. It is evident that there are at least three copies of RS1100 on the 25-kb insert of pUS1, none of which are located directly inside the 4.3-kb segment which harbors the *chq* genes (Fig. 5).

(g) RS1100 and genes encoding chlorohydroxyhydroquinone metabolism as DNA probes

The observations of instability of 2,4,5-T⁺ phenotype in wt AC1100 when grown under non-selective conditions are documented (Chatterjee et al., 1982) and several spontaneously emerging 2,4,5-T⁻ mutants have been isolated. Although, we recently

detected extensive deletion of chromosomal DNA in PT88 (Sangodkar et al., 1988), we do not know at this stage whether RS1100 is implicated in such rearrangements. Since *chq* genes of AC1100 are now available as a probe on a 4.8-kb *Bam*HI-*Xba*I fragment of pUS1028 along with RS1100 probe, we attempted to detect the presence of these sequences in various mutants of AC1100 as well as other *Pseudomonas* species. Fig. 7 shows the hybridization pattern of several mutants of AC1100 and other *Pseudomonas* strains using these two probes.

RS1100 hybridized only with the wt strain AC1100 and its mutants (Fig. 7B), confirming that these sequences are present in the wt as well as in the mutants. The results, however, do not help us to detect if any deletions near the site of insertion might have occurred in the mutants. Nevertheless, since uniform amounts of cultures were used for hybridization, dramatic variations in the intensity of hybridization to RS1100 probe demonstrate the presence of varying copy numbers of RS1100 in the mutants. In contrast, hybridization analysis (Fig. 7C) using the 4.8-kb *Bam*HI-*Xba*I probe confirms deletions having occurred in most of the 2,4,5-T⁻ mutants, including the Tn5-induced iso-

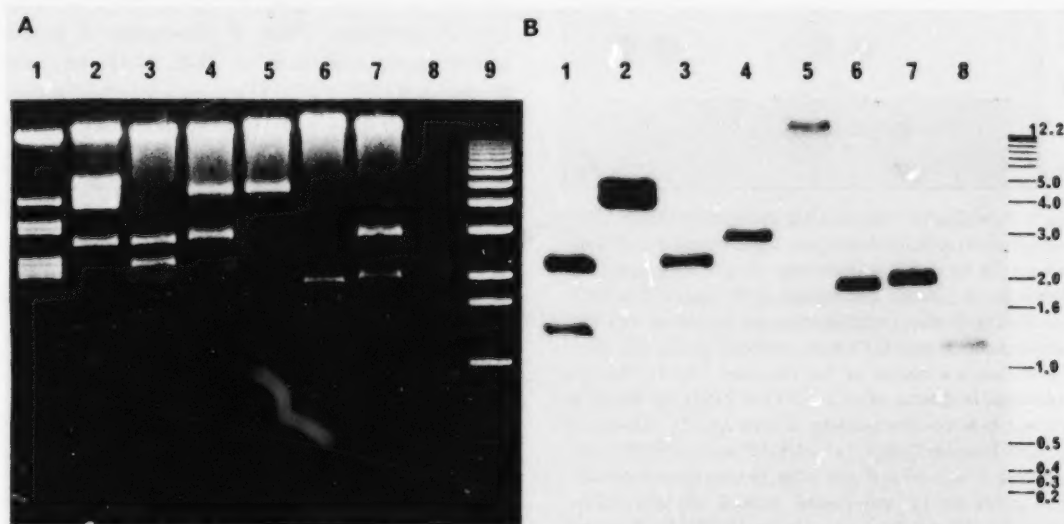


Fig. 6. Southern-blot analysis of cloned chromosomal fragments carrying 2,4,5-*t* genes using RS1100 DNA as probe. (Panel A) Agarose gel electrophoresis of restriction digests. Lanes: 1 and 2, pUS1 digested with *Sal*I or *Eco*RI, respectively; 3-7, pUS105 digested with *Eco*RI, *Hind*III + *Bam*HI, *Xba*I + *Bam*HI, *Xba*I + *Hind*III and *Xba*I + *Eco*RI, respectively; 8, 1.3-kb *Sal*I fragment of pPT165 as positive control; 9, DNA size marker (1-kb ladder, BRL). (Panel B) Autoradiograph of panel A hybridized with ^{32}P -labeled 1.3-kb *Sal*I fragment of pPT165.

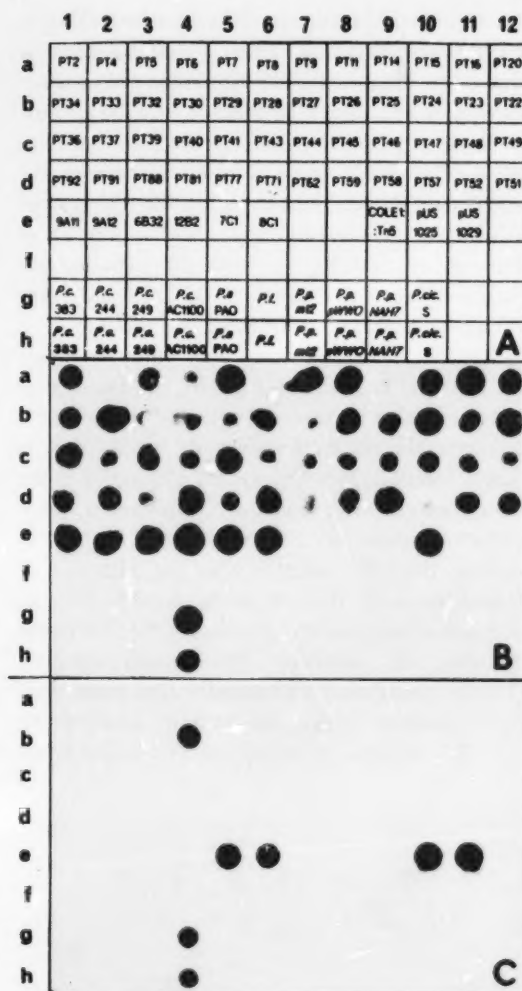


Fig. 7. Hybridization analysis using DNA probes. The cultures were grown to sufficient density and 100 μ l of each were filtered through the nitrocellulose membrane (BA85) using minifold I (Schleicher & Schuell) accommodating 96 samples. The DNA was fixed as in colony hybridization, and hybridized with ^{32} P-labeled probes. (Panel A) Cultures used for hybridization. Rows a to d contain a number of Tn5 generated 2,4,5-T⁻ mutants maintained in absence of 2,4,5-T (PT2 to PT92). Six strains in row e (1 to 6) are spontaneously isolated 2,4,5-T⁻ mutants of AC1100. Plasmids ColE1::Tn5, pUS1025 and pUS1029 (Table I and Fig. 5) were all in *E. coli* AC80. In rows g and h cultures were grown on LB and glucose minimal medium, respectively. *P.a.*, *P. aeruginosa*; *P.c.*, *P. cepacia*; *P.cic.*, *P. cichorii* (ATCC43388); *P.f.*, *P. fluorescens*; *P.p.*, *P. putida*. (Panel B) Hybridization of strains shown in panel A with 1.3-kb *SalI* fragment (RS1100) from pPT165 as probes. (Panel C) Hybridization of strains shown in panel A with the 4.8-kb *Bam*HI-*Xba*I fragment of pUS1028 (*chq* gene) as probe (Fig. 5).

lates. We have previously demonstrated that spontaneously isolated or Tn5-induced mutants of AC1100 are unstable, with gradual loss of phenotype (i.e., a 2,4,5-T⁻, trichlorophenol-positive mutant will acquire a 2,4,5-T⁻, trichlorophenol-negative phenotype, or a brown color producing mutant will become colorless in the long run). It is not clear whether such loss of phenotypes is due to point mutations or extensive deletions. Only PT30 which has the ability to revert to 2,4,5-T⁺ phenotype lighted up along with the spontaneous mutants, 7C1 and 8C1, which incidentally appear to be carrying a mutation in 2,4,5-T monooxygenase or uptake gene (R.A. Haugland, unpublished).

(h) Conclusions

Our results therefore demonstrate that the instability of 2,4,5-T degradation is caused by extensive deletions occurring in AC1100 maintained in absence of 2,4,5-T. In the present study we could clearly demonstrate the loss of at least the 4.8-kb segment carrying key degradative genes from most of the 2,4,5-T⁻ mutants. Whether such deletions occur because of the presence of RS1100 is difficult to predict. Interestingly, none of the other *Pseudomonas* species viz. *P. cepacia* 383, *P. cepacia* 244, *P. cepacia* 249, *P. aeruginosa* PAO, *P. fluorescens*, *P. putida* harboring plasmids such as TOL, NAH etc, and *P. cichorii* S (ATCC43388) shows any hybridization to RS1100 or the 4.8-kb segment containing the *chq* locus, suggesting that both these DNA segments are unique to the 2,4,5-T-degrading strain, *P. cepacia* AC1100. Whether RS1100 might have contributed to the recruitment of the 2,4,5-T genes from a non-pseudomonad ancestor is an open question, but the availability of these two nucleotide sequences as probes will allow us in future to conduct colony hybridization with environmental isolates to look for ancestry of these genes among natural microflora.

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REFERENCES

- Bagdasarian, M. and Timmis, K.N.: Host-vector systems for gene cloning in *Pseudomonas*. *Curr. Topics Microbiol.* 96 (1982) 47-67.
- Barsomian, G. and Lessie, T.G.: Replicon fusions promoted by insertion sequences on *Pseudomonas cepacia* plasmid pTGL6. *Mol. Gen. Genet.* 204 (1986) 273-280.
- Bolivar, R., Rodriguez, R.J., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S.: Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2 (1977) 95-113.
- Casse, F., Boucher, C., Julliot, J.S., Michael, M. and Denarie, H.: Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. *J. Gen. Microbiol.* 113 (1979) 229-242.
- Chakrabarty, A.M., Friello, D.A. and Bopp, L.H.: Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms. *Proc. Natl. Acad. Sci. USA* 75 (1978) 3109-3112.
- Chatterjee, D.K., Kellogg, S.T., Hamada, S. and Chakrabarty, A.M.: Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. *J. Bacteriol.* 146 (1981) 639-646.
- Chatterjee, D.K., Kilbane, J.J. and Chakrabarty, A.M.: Biodegradation of 2,4,5-trichlorophenoxyacetic acid in soil by pure culture of *Pseudomonas cepacia*. *Appl. Environ. Microbiol.* 44 (1982) 514-516.
- Chou, P. and Fasman, G.D.: Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47 (1978) 45-147.
- Comai, L. and Kosuge, T.: Transposable element that causes mutations in plant pathogenic *Pseudomonas* sp. *J. Bacteriol.* 154 (1983) 1162-1167.
- Darzens, A., Frantz, B., Vanags, R.I. and Chakrabarty, A.M.: Nucleotide sequence analysis of the phosphomannose isomerase gene (*pmi*) of *Pseudomonas aeruginosa* and comparison with the corresponding *Escherichia coli* gene *manA*. *Gene* 42 (1986) 293-302.
- Deretic, V., Tomasek, P., Darzens, A. and Chakrabarty, A.M.: Gene amplification induces mucoid phenotype in *rec-2 Pseudomonas aeruginosa* exposed to Km. *J. Bacteriol.* 165 (1986) 510-516.
- Figurski, D. and Helinski, D.R.: Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc. Natl. Acad. Sci. USA* 76 (1979) 1648-1652.
- Fishman, S.F., Rosteck Jr., P.R., and Hershberger, C.L.: A 2.2 kilobase repeated DNA segment is associated with DNA amplification in *Streptomyces fradiae*. *J. Bacteriol.* 161 (1985) 199-206.
- Frantz, B., Ngai, K.-L., Chatterjee, D.K., Ornston, L.N. and Chakrabarty, A.M.: Nucleotide sequence and expression of *clcD*, a plasmid-borne diene lactone hydrolase gene from *Pseudomonas* sp. *J. Bacteriol.* 169 (1987) 704-709.
- Gay, P., Le Coq, D., Steinmetz, M., Berkelman, T. and Kado, C.I.: Positive selection procedure for entrapment of insertion sequence elements in Gram-negative bacteria. *J. Bacteriol.* 129 (1985) 918-921.
- Grindley, N.D.F. and Reed, R.R.: Transpositional recombination in prokaryotes. *Annu. Rev. Biochem.* 54 (1985) 863-896.
- Harshey, R.M., Getzoff, E.D., Baldwin, D.L., Miller, J.L. and Chaconas, G.: Primary structure of phase Mu transposase: homology to Mu repressor. *Proc. Natl. Acad. Sci. USA* 82 (1985) 7676-7680.
- Hohn, B. and Collins, J.: A small cosmid for efficient cloning of large DNA fragments. *Gene* 11 (1980) 291-298.
- Jorgensen, R.A., Rothstein, S.J. and Reznikoff, W.S.: A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* 177 (1979) 65-72.
- Kaluza, K., Hahn, M. and Hennecke, H.: Repeated sequences similar to insertion elements clustered around the *nif* region of the *Rhizobium japonicum* genome. *J. Bacteriol.* 162 (1985) 535-542.
- Kilbane, J.J., Chatterjee, D.K., Karns, J.S., Kellogg, S.T. and Chakrabarty, A.M.: Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. *Appl. Environ. Microbiol.* 44 (1982) 72-78.
- Kleckner, N.: Transposable elements in prokaryotes. *Annu. Rev. Genet.* 15 (1981) 341-404.
- Maniatis, T., Fritsch, E.F. and Sambrook, J.: *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
- Mazodier, P., Cossart, P., Giraud, E. and Gasser, F.: Completion of the nucleotide sequence of the central region of Tn5 confirms the presence of three resistance genes. *Nucleic Acids Res.* 13 (1985) 195-205.
- Negoro, S., Taniguchi, T., Kanaoka, M., Kimura, H. and Okada, H.: Plasmid-determined enzymatic degradation of nylon oligomers. *J. Bacteriol.* 155 (1983) 22-31.
- Pabo, C.O. and Sauer, R.T.: Protein-DNA recognition. *Annu. Rev. Biochem.* 53 (1984) 293-321.
- Poustka, A., Rackwitz, H.-R., Frischauf, A.-M., Hohn, B. and Lehrach, H.: Selective isolation of cosmid clones by homologous recombination in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 81 (1984) 4129-4133.

- Sangodkar, U.M.X., Chapman, P.J. and Chakrabarty, A.M.: Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T in *Pseudomonas cepacia* AC1100. *Gene* 71 (1988) 267-277.
- Sapienza, C., Rose, M.R. and Doolittle, W.F.: High-frequency genomic rearrangements involving archaebacterial repeat sequence elements. *Nature* 299 (1982) 182-185.
- Scordilis, G.E., Ree, H. and Lessie, T.G.: Identification of transposable elements which activate gene expression in *Pseudomonas cepacia*. *J. Bacteriol.* 169 (1987) 8-13.
- Szabo, L.J. and Mills, D.: Integration and excision of pMC7105 in *Pseudomonas syringae* pv. *phaseolicola*: involvement of repetitive sequences. *J. Bacteriol.* 157 (1984) 821-827.
- Tomasek, P., Frantz, B., Chatterjee, D.K. and Chakrabarty, A.M.: Genetic and molecular basis of the microbial degradation of herbicides and pesticides. In Augustine, P.C., Danforth, H.-D. and Bakst, M.R. (Eds.), *Biotechnology for Solving Agricultural Problems*. Martinus Nijhoff, Dordrecht, 1986, pp. 355-368.

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